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(54) GENETICALLY MODIFIED PLANT CAPABLE OF BIOSYNTHESIZING CAPSINOID

(57) The present invention provides a genetically modified plant that biosynthesizes an increased amount of capsinoids, a method of producing the genetically modified plant, and a production method of capsinoids from the genetically modified plant.

More particularly, the present invention provides a genetically modified plant capable of producing capsinoids, which shows a decreased expression or activity of an enzyme that catalyzes an amino group conversion reaction from vanillin to vanillylamine as compared to wild strains, and the like. As a method of suppressing expression or activity of an enzyme, an introduction of DNA encoding an antisense RNA, iRNA, ribozyme or dominant-negative mutant for the target gene, a destruction of the gene by a knockout method or mutagen treatment, an introduction of a gene encoding an antibody against the enzyme and the like can be mentioned.

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Description

Technical Field

[0001] The present invention relates to a genetically modified plant that biosynthesizes an increased amount of capsinoids, a method of producing the genetically modified plant, a production method of capsinoids from the genetically modified plant and the like.

Background Art

[0002] Capsaicinoid (capsaicin, dihydrocapsaicin etc.), which is a pungent component of chili pepper, is effective for preventing obesity, since it promotes energy metabolism, stimulates central nerves to promote hormone secretion and activates lipolytic enzyme. In addition, it sterilizes gastrointestinal tract and improves immunity, and also effective for activating immunity and relieving fatigue. However, since capsaicinoid irritates mucosa, ingestion in large amounts may upset the gastrointestinal condition. Moreover, many Japanese people do not like pungent tastes.

[0003] As less pungent chili pepper, "CH-19 Sweet" (Variety Registration No. 10375), which is a nonpungent fixed variety of chili pepper selected and fixed by Yazawa et al. has been reported to contain a large amount of novel capsinoids (e.g., non-patent document 1). Such compounds belonging to capsinoids (fatty acid ester of vanillyl alcohol, capsiate, dihydrocapsiate etc., hereinafter sometimes to be simply referred to as "capsinoid" or "capsinoids") are different from capsaicinoid and do not have a pungent taste. However, they have been reported to show an immunity enhancing action, an energy metabolism activation action, oxygen consumption promoting action and the like (e.g., patent documents 1 and 2, and non-patent document 2), and are expected to be starting materials of supplement and the like in the future.

[0004] In most chili peppers, vanillylamine is formed from phenylalanine via ferulic acid, vanillin and the like, and capsaicinoid is produced from vanillyamine and branched chain fatty acid by capsaicinoid synthase (Fig. 1(a)). In contrast, capsaicinoid is scarcely produced in CH-19 Sweet and capsinoid is produced instead. However, the reason therefor has been unknown for years.

[0005] As mentioned above, capsinoids have a little pungent taste, but shows superior physiological activities similar to those of capsaicinoid. Therefore, future application as a starting material of supplements and the like is expected. Therefore, it is desired to clarify the biosynthesis pathway of capsinoids with the aim of breeding and development of new plant varieties capable of producing capsinoids.

[prior art references]

[patent documents]

[0006]

patent document 1: JP-A-11-246478
patent document 2: JP-A-2001-026538

[non-patent document]

[0007]

non-patent document 1: Yazawa et al., Journal of the Japanese Society for Horticultural Science, vol. 58, pages 601-607, 1989
non-patent document 2: Biosci. Biotech. Biochem., 65 (12), 2735-2740 (2001)

[SUMMARY OF THE INVENTION]

Problems to be Solved by the Invention

[0008] Therefore, an object of the present invention is to clarify the biosynthesis pathway of capsinoids and, based on the finding, provide a plant capable of producing capsinoids by genetic modification from a plant other than CH-19 Sweet currently known to produce capsinoids.

Means of Solving the Problems

[0009] Present inventors have conducted intensive studies to achieve the above-mentioned object and found that, in CH-19 Sweet, pAMT gene encoding aminotransferase that catalyzes an amino group conversion reaction from vanillin to vanillylamine contains an insertional mutation. That is, it has been clarified as regards CH-19 Sweet that a stop codon is produced due to an insertional mutation of one base in the coding region of pAMT gene of pungent variety "CH-19 Hot", which is a parental variety thereof, and functional aminotransferase is not produced. The present inventors made a hypothesis that, as a result of the mutation of the pAMT gene, a reduction reaction from vanillin to vanillyl alcohol becomes dominant in CH-19 Sweet instead of the amino group conversion from vanillin to vanillylamine, and a further esterification reaction with branched chain fatty acid results in capsinoids production (Fig. 1(b)).
To verify the hypothesis, present inventors have prepared transgenic plants having a pAMT antisense nucleic acid introduced into a pungent variety, Capsicum annuum variety 'Takanotsume'. It has been found that production of capsaicinoid decreases but production of capsinoid markedly increases in a lineage showing remarkably decreased expression of pAMT gene from among the obtained transgenic plants.
Present inventors have conducted further studies based on these findings and completed the present invention.
[0010] Accordingly, the present invention provides the following:

[1] a genetically modified plant capable of producing capsinoids, which shows a decreased expression or activity of an enzyme that catalyzes an amino group conversion reaction from vanillin to vanillylamine as compared to wild strains;
[2] the plant described in the above-mentioned [1], wherein the aforementioned enzyme is a pAMT gene product;
[3] the plant described in the above-mentioned [1] or [2], wherein the aforementioned decreased expression or activity of the enzyme is caused by a destruction or mutation of the enzyme gene, a decomposition or suppressed translation of a transcription product of the gene, or inhibition of action of the enzyme on vanillin;
[4] the plant described in any of the above-mentioned [1] to [3], wherein the wild strain has a capsaicinoid biosynthesis system;
[5] the plant described in the above-mentioned [4], wherein the wild strain is a plant belonging to the genus Capsicum;
[6] a method of producing a genetically modified plant capable of producing capsinoids, comprising performing a genetic modification to decrease expression or activity of an enzyme that catalyzes an amino group conversion reaction from vanillin to vanillylamine in a plant having a capsaicinoid biosynthesis system;
[7] the method described in the above-mentioned [6], wherein the genetic modification is selected from the group consisting of an introduction of DNA encoding an antisense RNA, iRNA, ribozyme or dominant-negative mutant for the aforementioned enzyme gene, a destruction of the gene by a knockout method or mutagen treatment, and an introduction of a gene encoding an antibody against the enzyme;
[8] the method described in the above-mentioned [6] or [7], wherein the aforementioned enzyme is a pAMT gene product;
[9] the method described in any of the above-mentioned [6] to [8], wherein the plant having a capsaicinoid biosynthesis system belongs to the genus Capsicum; and
[10] a method of producing capsinoid, comprising recovering the capsinoid from a fruit of the plant described in any of the above-mentioned [1] to [5].

Effect of the Invention

[0011] The present invention enables to increase production of capsinoids also in a plant variety that substantially does not produce capsinoids or produces only a small amount thereof.

Brief Description of the Drawings

[0012]

Fig. 1 shows expected synthesis pathways of capsaicin (a) and capsiate (b).
Fig. 2 is a schematic diagram of Ti-plasmid vector, pIG121-Hm, having pAMT gene inserted in the antisense direction.
Fig. 3 shows the results of genomic PCR using DNA extracted from leaf piece(s) of transformed chili pepper as a template, wherein M is a marker, C is a control (Capsicum annuum variety 'Takanotsume' without genetic modification), and each number shows selected transformed chili pepper individuals.
Fig. 4 shows the analytical results of capsiate content (a) and capsaicin content (b) of fruits of transformed chili pepper and CH-19 Sweet, and Capsicum annuum variety 'Takanotsume'.
Fig. 5 shows the analytical results of transcription expression of pAMT gene in fruits of transformed chili pepper and

Capsicum annuum variety 'Takanotsume'.

Fig. 6 shows the analysis results of proteins extracted from fruits of transformed chili pepper, by Western blot analysis, wherein each lane is 1: CH-19 Hot, 2: TG-1, 3: TG-3, 4: TG-8, 5: TG-14, 6:CH-19 Sweet.

[Mode for Carrying out the Invention]

[0013] The present invention provides a genetically modified plant capable of producing capsinoids as compared to wild strains, which shows a decreased expression or activity of an enzyme that catalyzes an amino group conversion reaction from vanillin to vanillylamine.

[0014] In the present invention, the "wild strain" means a plant to be the target of genetic modification in the present invention. The wild strain to be used in the present invention may be any as long as it functionally expresses enzyme group (e.g., phenylalanineammonialyase (PAL), cinnamic acid 4-hydrolase (Ca4H), coumaric acid 3-hydrolase (Ca3H), caffeic acid O-methyltransferase (COMT), capsaicinoid synthase (CS)) necessary for biosynthesis of capsaicinoid. The plant may have such enzyme group by nature, or one or more enzymes may be provided by the expression of a foreign gene. Preferably, the wild strain that can be used for the present invention is a plant having capsaicinoid production ablity by nature, more preferably, plants belonging to Capsicum. Examples of thereof include, but are not limited to, C. annuum (variety 'Takanotsume', CH-19 Hot, Yatsufusa', 'Toranoo', 'Fushimiama', and the like), C. baccatum (aji amarillo and the like), C. chinense (habanero pepper, Bhut Jolokia and the like), C. frutescens (Capsicum frutescens L. and the like), and C. pubescens (rocoto and the like).

[0015] An enzyme that catalyzes an amino group conversion reaction from vanillin to vanillylamine is not particularly limited as long as it is a protein having an aminotransferase activity and produced by the above-mentioned wild strain, which can use vanillin as a substrate. Preferred are aminotransferase pAMT (Putative Aminotransferase) gene having high homology with GABA aminotransferase from rice or tomato, which was found in habanero pepper, and aminotransferase encoded by its ortholog in other plant variety.

[0016] More specifically, pAMT protein (also referred to as pAMT gene product) of the present invention is a protein containing the same or substantially the same amino acid sequence as the amino acid sequence shown by SEQ ID NO: 2, and having an aminotransferase activity at least equivalent (e.g., 0.5-fold or more, preferably 0.7-fold or more) to the protein consisting of the amino acid sequence shown by SEQ ID NO: 2. Here, the "substantially the same amino acid sequence" means an amino acid sequence of natural allele variant or gene polymorphism of pAMT protein derived from CH-19 Hot consisting of the amino acid sequence shown by SEQ ID NO: 2, and an amino acid sequence of its ortholog in other plant variety and the like. Examples of the ortholog of pAMT in other plant variety include pAMT derived from habanero pepper (Refseq No. AAC78480), GABA aminotransferase from rice (Refseq No. AAQ14479) and GABA aminotransferase from tomato (Refseq No. AA092257) and the like can be mentioned.

[0017] The above-mentioned "substantially the same amino acid sequence" desirably has not less than 80%, preferably not less than 90%, more preferably not less than 95%, most preferably not less than 97%, of similarity (preferably, identity) to the amino acid sequence shown by SEQ ID NO: 2. The "similarity" here means, when two amino acid sequences were aligned using mathematical algorithm known in the art, a proportion (%) of the same and similar amino acid residues to the total overlapping amino acid residues in an optimal alignment (preferably, the algorithm can consider introduction of gap(s) into one or both of the sequences for an optimal alignment). The similarity of amino acid sequences in the present specification can be calculated using homology calculation algorithm NCBI BLAST (National Center for Biotechnology Information Basic Local Alignment Search Tool) under the following conditions (expectancy =10; allow gap; matrix =BLOSUM62; filtering=OFF).

[0018] In the present invention, an enzyme group of capsinoid biosynthesis system is at least partly common with an enzyme group constituting a capsaicinoid biosynthesis system, except those that catalyze the reduction reaction from vanillin to vanillyl alcohol, and the present invention is based on the finding that the quantitative ratio of the produced capsinoid and capsaicinoid depends on which of the above-mentioned reduction reaction and an amino group conversion reaction from vanillin to vanillylamine proceeds more dominantly. Therefore, by lowering the expression or activity of an enzyme that catalyzes an amino group conversion reaction from vanillin to vanillylamine than that of wild strains, the reduction reaction from vanillin to vanillyl alcohol becomes more dominant and, as a result, the capsinoid production capability can be improved.

[0019] The "expression" of an enzyme that catalyzes an amino group conversion reaction from vanillin to vanillylamine means that a translation product (i.e., protein) is produced by a gene encoding the enzyme (e.g., pAMT gene etc.), and is localized at the action site thereof in a functional state. The "lower expression" of an enzyme means a result of a significantly decreased protein amount of the enzyme present in a genetically modified plant, as compared to that of wild strain. Therefore, a genetic modification to lower enzyme expression can be performed in any stage, for example, at a gene level, transcription level, post-transcription regulation level, translation level, post-translational modification level and the like.

[0020] For example, as a genetic modification that suppresses enzyme expression at a gene level, destruction of the enzyme gene and introduction of functionally defective mutation can be mentioned. Here, the "functionally defective mutation" may be a mutation that decreases the enzyme activity such that the capsinoid production increases significantly as compared to wild strains, and a mutation that completely eliminates the enzyme activity is not necessarily intended. Such genetic modification can be achieved by a knockout (or knockin) technique of an enzyme gene utilizing a homologous recombination. As a specific means to knockout an enzyme gene, a method including isolating an enzyme gene (genome DNA) derived from a plant to be a target of genetic modification (i.e., wild strain) by a conventional method (e.g., in the case of a pAMT gene, pAMT genome DNA can be cloned by a colony or plaque hybridization method from genome DNA library prepared from genomic DNA isolated from a wild strain, using a nucleic acid containing whole or part of the base sequence of CH-19 Hot-derived pAMT cDNA shown by SEQ ID NO: 1 as a probe) and inserting a DNA chain (targeting vector) having a DNA sequence constructed to inactivate the gene into the enzyme gene locus of a wild strain by homologous recombination and the like can be preferably used. A DNA chain (targeting vector) having a DNA sequence constructed to inactivate the gene is prepared by, for example,

(1) inserting other DNA fragment (e.g., selection marker gene of drug resistance gene, reporter gene and the like) into the exon region or promoter region thereof to destroy the exon or promoter function,
(2) cleaving out whole or part of the enzyme gene by using the Cre-loxP system or Flp-frt system to delete the gene,
(3) inserting a stop codon into a protein coding region to prevent complete translation of protein, or
(4) inserting a DNA sequence (e.g., polyadenylated signal and the like) that terminates gene transcription into a transcription region to prevent synthesis of complete mRNA.

The selection marker gene is preferably in the form of an expression cassette containing any promoters capable of intracellularly functioning in a target plant. When the gene is inserted such that it is placed under control of an endogenous promoter of the target enzyme gene, a selection marker gene does not require a promoter.

[0021] Generally, gene recombination in plant is mostly nonhomologous, and introduced DNA is randomly inserted into any position of chromosome. Therefore, efficient selection of only a clone targeted to an enzyme gene by homologous recombination, cannot be achieved on the basis of selection of detection of drug resistance, expression of reporter gene and the like. Thus, confirmation of recombination site of any selected clone by Southern hybridization method or PCR method becomes necessary. Thus, when, for example, the Herpes simplex virus-derived thymidine kinase (HSV-tk) gene that imparts gancyclovir sensitivity is ligated outside the region homologous to the target sequence in a targeting vector, a cell having the vector randomly inserted therein has an HSV-tk gene, and therefore, the cell cannot grow in a medium containing gancyclovir. However, a cell targeted to an enzyme gene locus by homologous recombination becomes gancyclovir resistant since it does not have an HSV-tk gene and can be selected. Alternatively, when, for example, a diphtheria toxin gene is ligated instead of the HSV-tk gene, since a cell having the vector randomly inserted therein is killed by the toxin it produces, a homologous recombinant can also be selected in the absence of a drug. The presence of introduced DNA can be confirmed by applying a part of the formed resistant colony to PCR or Southern hybridization.

[0022] As another genetic modification to suppress enzyme expression at a gene level, introduction of a functionally defective mutation into an enzyme gene by a mutagen treatment can be mentioned. As the mutagen treatment, any can be used as long as it induces point mutation, deletion or frame shift mutation in the DNA of a wild strain. Specifically, treatments with ethylnitrosourea, nitrosoguanidine, benzopyrene, acridine dye, radiation and the like can be mentioned. In addition, various alkylating agents and carcinogens can also be used as mutagens. As a method of reacting a mutagen with a cell, the method described in Technique of Tissue Culture, 3rd edition (Asakura Publishing Co., Ltd.), The Japan Tissue Culture Association ed. (1996), Nature Genet., 314 (2000) and the like can be used. A functionally defective variant of an enzyme gene can be selected using the amount of target protein as an index. In the case of pAMT, for example, Western blot analysis can be performed using a polyclonal antibody to pAMT protein, and the amount of the pAMT protein can be determined. As for a plant showing a significantly decreased pAMT protein level, introduction of a mutation into the gene can be confirmed by determining the nucleotide sequence of the enzyme gene isolated from a part of the plant.

[0023] As a genetic modification to suppress enzyme expression at a translation level, introduction of a nucleic acid having an activity to degrade a transcription product of an enzyme gene, or a nucleic acid that suppresses translation of a transcription product into an enzyme protein can be mentioned. As such nucleic acid, a nucleic acid containing a nucleotide sequence complementary or substantially complementary to the nucleotide sequence of mRNA of the enzyme, or a part of the sequence, can be mentioned.

The nucleotide sequence substantially complementary to the nucleotide sequence of mRNA of a target enzyme means a nucleotide sequence having complementation of the level capable of binding to the target sequence of the mRNA and inhibiting the translation thereof under physiological conditions in the subject plant. Specifically, for example, a nucleotide sequence having a similarity of not less than about 80%, preferably not less than about 90%, more preferably not less than about 95%, most preferably not less than about 97%, to the region overlapping with a nucleotide sequence completely complementary to the nucleotide sequence of the mRNA (i.e., nucleotide sequence of mRNA complementary chain).

The "similarity of nucleotide sequence" in the present invention can be calculated using homology calculation algorithm NCBI BLAST (National Center for Biotechnology Information Basic Local Alignment Search Tool) and under the following conditions (expectancy =10; allow gap; filtering=ON; match score=1; mismatch score=-3).

[0024] More specifically, for example, when the enzyme is pAMT, examples of a nucleotide sequence complementary or substantially complementary to the nucleotide sequence of pAMT mRNA include

(a) the nucleotide sequence shown by SEQ ID NO: 1 or
(b) a nucleotide sequence that hybridizes to the nucleotide sequence under stringent conditions, which is a nucleotide sequence complementary or substantially complementary to a sequence encoding a protein having an activity equivalent to that of a protein consisting of the amino acid sequence shown by SEQ ID NO: 2. Under stringent conditions means, for example, the conditions described in Current Protocols in Molecular Biology, John Wiley & Sons, 6.3.1-6.3.6, 1999, for example, hybridization with 6×SSC (sodium chloride/sodium citrate)/45°C, then washing one or more times with 0.2×SSC/0.1% SDS/50 - 65°C and the like, and those of ordinary skill in the art can appropriately select hybridization conditions affording stringency equivalent thereto.

[0025] pAMT mRNA is preferably pAMT mRNA of CH-19 Hot containing the nucleotide sequence shown by SEQ ID NO: 1, or ortholog thereof in other plant variety (e.g., habanero pepper-derived pAMT (Refseq No. AF085149), GABA aminotransferase of rice (Refseq No. AF297651) and GABA aminotransferase of tomato (Refseq No. AY240231) etc.), or further, natural allele variant, or gene polymorphism thereof.

[0026] The "part of a nucleotide sequence complementary or substantially complementary to nucleotide sequence of mRNA of a target enzyme" is not particularly limited in terms of length and position, as long as it can specifically bind to target enzyme mRNA and inhibit translation of protein from the mRNA. From the aspect of sequence specificity, however, it contains at least 10 bases, preferably not less than about 15 bases, more preferably not less than about 20 bases, of a part complementary or substantially complementary to the target sequence.

[0027] Specifically, preferable examples of the nucleic acid containing a nucleotide sequence complementary or substantially complementary to a nucleotide sequence of mRNA of a target enzyme, or a part of such sequence, include the following (a) to (c).

(a) antisense RNA to target enzyme mRNA
(b) iRNA (interfering RNA) to target enzyme mRNA
(c) ribozyme to target enzyme mRNA

(a) antisense RNA to target enzyme mRNA

[0028] The "antisense RNA" in the present invention is a nucleic acid containing a nucleotide sequence complementary or substantially complementary to the nucleotide sequence of target mRNA, or a part thereof, and has a function to suppress protein synthesis by forming a specific and stable double strand with target mRNA. The target region of antisense RNA is not particularly limited in terms of length, as long as hybridization of the antisense RNA results in the inhibition of translation of target mRNA into an enzyme protein, and it may be the total mRNA sequence encoding the enzyme or a partial sequence thereof, wherein a shorter one is about 10 bases long and a longer one is the total mRNA sequence.

Moreover, the antisense RNA of the present invention may not only hybridize to target enzyme mRNA to inhibit translation into a protein, but also be bound to a gene of the enzyme, which is a double stranded DNA, to form a triplex and inhibit transcription to RNA (anti-gene).

(b) iRNA to target enzyme mRNA

[0029] In the present specification, a double stranded RNA consisting of oligoRNA complementary to target enzyme mRNA and a complementary chain thereof, i.e., iRNA, is also defined to be encompassed in the nucleic acid containing a nucleotide sequence complementary or substantially complementary to a nucleotide sequence of mRNA of a target enzyme, or a part thereof. A phenomenon of intracellular introduction of a double stranded RNA resulting in degradation of mRNA complementary to RNA thereof, so-called RNA interference (RNAi), has long been known in nematode, insect, plant and the like. Ever since this phenomenon was confirmed to widely occur in animal cells [Nature, 411(6836): 494-498 (2001)], it has been widely used as an alternative technique of ribozyme. iRNA can be appropriately designed based on the information of nucleotide sequence of mRNA to be the target and using commercially available software (e.g., RNAi Designer; Invitrogen).

(c) ribozyme to target enzyme mRNA

[0030] As ribozyme with broadest utility, self-splicing RNA seen in infectious RNA such as viroid, virusoid and the like can be mentioned, and hammerhead type, hairpin type and the like are known. The hammerhead type shows enzyme activity with only about 40 bases, and it is possible to specifically cleave target mRNA alone by rendering several bases on both ends (about 10 bases in total) adjacent to the part with a hammerhead structure form a sequence complementary to a desired cleavage site of mRNA. Since this type of ribozyme contains RNA alone as a substrate, it is further advantageous since it does not attack genomic DNA. When target enzyme mRNA itself has a double stranded structure, the target sequence can be converted to a single strand by using a hybrid ribozyme wherein virus nucleic acid-derived RNA motif capable of specifically binding to RNA helicase is ligated [Proc. Natl. Acad. Sci. USA, 98(10): 5572-5577 (2001)]. Moreover, to promote transfer to the cytoplasm, a hybrid ribozyme wherein a sequence obtained by modifying tRNA is further ligated may be used [Nucleic Acids Res., 29(13): 2780-2788 (2001)].

[0031] A DNA encoding an antisense RNA to mRNA of target enzyme, when the antisense RNA is comparatively long and is, for example, not less than 100 bp, can be produced by cloning cDNA of the target enzyme according to a conventional method, and directly ligating same, or ligating after fragmentation into a desired length containing a desired region by digestion with a suitable restriction enzyme, to the downstream of a promoter capable of functioning in the cell of the target plant, in an antisense direction. When the antisense RNA is comparatively short and is, for example, 100 bp or below, it can also be chemically synthesized by a commercially available DNA/RNA automatic synthesizer.

DNA encoding iRNA to target enzyme mRNA can be constructed as a DNA encoding hairpin type dsRNA by amplifying the target sequence (for example, about 200 - 500 bases) on mRNA by RT-PCR to give double stranded DNA, and ligating two double stranded DNAs in the sense direction and antisense direction via suitable linker sequences by using restriction enzyme and ligase. The linker sequence is not particularly limited in terms of its sequence and length as long as it can form a loop capable of forming a hairpin type dsRNA on transcription of the constructed DNA. For example, when a reporter gene such as β-glucuronidase gene is used as a linker sequence, cutting out iRNA from the hairpin type dsRNA can be monitored by detection of the expression of the reporter gene.

The DNA encoding ribozyme to target enzyme mRNA can be prepared by synthesizing a DNA having a nucleotide sequence of designed ribozyme by a DNA/RNA automatic synthesizer.

[0032] A nucleic acid containing a nucleotide sequence complementary or substantially complementary to a nucleotide sequence of mRNA of a target enzyme, or a part thereof, may be introduced into a target plant by any genetic engineering method. For example, infection with a plant virus containing a virus genome genetically engineered to contain the nucleic acid, and transformation of a target plant cell with an expression vector containing the nucleic acid can be mentioned.

Preferably, the genetically modified plant of the present invention is a transgenic plant obtained by transforming the cell of the wild strain with an expression vector containing the above-mentioned nucleic acid under control of a promoter capable of functioning in a target plant (wild strain) cell.

[0033] The promoter capable of functioning in a wild strain cell can be appropriately selected according to the plant variety used as the wild strain. Generally, a gene promoter constitutionally expressed in a plant cell, preferably constitutional promoter derived from a plant or plant virus (e.g., Cauliflower mosaic virus (CaMV) 35S promoter, CaMV 19S promoter, NOS promoter etc.), or cis promoter in a wild strain target enzyme gene and the like can be mentioned.

[0034] In the expression vector of the present invention, a nucleic acid containing a nucleotide sequence complementary or substantially complementary to a nucleotide sequence of mRNA of a target enzyme, or a part thereof is located at the downstream of a promoter capable of functioning in a wild strain cell, so that transcription thereof can be regulated by the promoter. It is preferable that a transcription termination signal (terminator region) capable of functioning in a wild strain be further added at the downstream of the nucleic acid sequence. Examples of the terminator include NOS (nopalinesynthase) gene terminator and the like.

[0035] The expression vector of the present invention may further contain a cis-regulatory element such as an enhancer sequence and the like. Moreover, the expression vector desirably further contains a marker gene for selection of a transformant such as a drug resistance gene marker and the like [e.g., neomycin phosphotransferase II (NPTII) gene, hygromycin phosphotransferase (HPT) phosphinothricinacetyltransferase (PAT) gene, glyphosate resistance gene and the like.

To facilitate large-scale preparation and purification, moreover, the expression vector desirably contains a replication origin that enables autonomous replication in Escherichia coli and a selection marker gene in Escherichia coli (for example, ampicillin resistance gene, tetracycline resistance gene etc.). The expression vector of the present invention can be conveniently constructed by inserting an expression cassette of the above-mentioned nucleic acid and, where necessary, a selection marker gene into a cloning site of a pUC or pBR Escherichia coli vector.

[0036] When the above-mentioned nucleic acid is introduced by utilizing infection with Agrobacterium tumefaciens or Agrobacterium rhizogenes, an expression cassette of the nucleic acid can be inserted into the T-DNA region (region to be transferred to plant chromosome) on Ti or Ri plasmid retained by the bacterium, and put to use. For a standard method of transformation by Agrobacterium method, a binary vector system is used. The function necessary for T-DNA transfer is independently supplied by both T-DNA itself and Ti (or Ri) plasmid, and each constituent factor can be divided on different vectors. A binary plasmid has a 25 bp border sequence on both ends necessary for cutting out and insertion of T-DNA, and is free of a plant hormone gene causing crown gall (or hairy root), thus simultaneously affording a room for insertion of a foreign gene. As such binary vector, for example, pBI101, pBI121 (both CLONTECH) and the like are commercially available. The vir region that acts on insertion of T-DNA is located on a different Ti (or Ri) plasmid called a helper plasmid.

[0037] Conventionally known various methods can be used for transformation of plants. Examples thereof include a method including isolating protoplast from a cell of a target plant by a treatment with a cell wall degrading enzyme such as cellulose, hemicellulose and the like, and adding polyethylene glycol to a suspension of the protoplast and an expression vector containing the above-mentioned pAMT gene expression cassette to insert the expression vector into the protoplast by an endocytosis-like process (PEG method), a method including inserting an expression vector into a lipid membrane vesicle such as phosphatidylcholine and the like by sonication and the like, and fusing the vesicle and protoplast in the presence of PEG (liposome method), a method including fusion by a similar process using a mini-cell, and a method including applying an electric pulse to a suspension of protoplast and expression vector to insert the vector in the extracellular solution into the protoplast (electroporation method). However, these methods are complicated since a culture technique for redifferentiation from the protoplast to a plant is necessary. As methods of transgene into an intact cell having a cell wall, a microinjection method including pushing a micropipette into a cell, and intracellularly injecting a vector DNA in the pipette by hydraulic pressure or gas pressure, a direct introduction method including accelerating metal microparticles coated with DNA by powder explosion or gas pressure for intracellular introduction such as a particle gun method and the like, and a method utilizing infection with Agrobacterium can be mentioned. Microinjection has a defect in that the operation requires practice, and the number of handleable cells is small. Considering the convenience of operation, therefore, plant is preferably transformed by an Agrobacterium method or a particle gun method. The particle gun method is further useful since a gene can be directly introduced into the apical meristem of a plant before harvesting. Moreover, in the Agrobacterium method, by simultaneously inserting a plant virus, for example, genomic DNA of geminivirus such as tomato golden mosaic virus (TGMV) and the like between border sequences of a binary vector, virus infection spreads over the whole plant by merely inoculating a cell suspension with a syringe barrel and the like to a cell at any part of a plant before harvesting, and an object gene is simultaneously introduced into the whole plant.

[0038] In the particle gun method and Agrobacterium method, however, since transgene is often chimeric, a sample cell that permits introduction of the above-mentioned nucleic acid into a germ line cell at high frequency needs to be used for transformation. For example, embryo, hypocotyl piece, embryogenic callus, isolated growing point and the like can be mentioned. On the other hand, since the above-mentioned transformation method using protoplast and microinjection method permit selection of a redifferentiation plant from a single cell, they are superior transformation means for obtaining a homogeneous transgenic plant having a pAMT gene introduced into the whole cell.

[0039] As specific examples, introduction of an object nucleic acid by Agrobacterium and regeneration method of transformed cell into a plant in a Capsicum plant are shown in the following, which are mere examples and do not at all limit the production method of the genetically modified plant of the present invention.

A seed of a chili pepper plant is sterilized, sown on a suitable seed germination medium (e.g., MS medium, LS medium, B5 medium and the like), and aseptically grown. On the other hand, Agrobacterium having a promoter connected with an object nucleic acid, and transformed with a plasmid having a kanamycin resistance gene and a hygromycin resistance gene is cultured, and diluted with a suitable medium to give an Agrobacterium solution. Cotyledon after germination is immersed in the Agrobacterium solution for about 10 min, transplanted to a selection medium (e.g., MS medium, LS medium, B5 medium and the like) added with a desired plant hormone (e.g., auxins such as IAA, NAA and the like, cytokinins such as kinetin, benzyladenine and the like) and kanamycin and hygromycin antibiotics, and cultivated at 20 - 30°C. The obtained kanamycin and hygromycin resistant callus and seedling are transplanted to a redifferentiation (rooting) medium (e.g., MS medium, LS medium, B5 medium and the like) added with benzyladenine as necessary to induce redifferentiation (rooting), whereby a seedling plant is obtained. When several true leaves are observed, the seedling plant is transplanted to the soil for acclimation, and grown in a controlled greenhouse such as biotron and the like. Seed and fruit can be obtained from the thus-obtained transformant.

[0040] A genomic DNA is extracted from the above-mentioned transformant according to a conventional method, the DNA is cleaved with a suitable restriction enzyme, and Southern hybridization is performed using the introduced object nucleic acid as a probe, whereby the presence or absence of transformation can be confirmed. In addition, the presence or absence of transformation can also be confirmed by synthesizing a primer that specifically amplifies the object nucleic acid and performing a PCR method.

Moreover, RNA can be extracted from a transformant or untransformant by a conventional method, and changes in the expression level of target enzyme mRNA can be examined by quantitative RT-PCR, Northern hybridization and the like. Alternatively, a protein can be extracted from a transformant or untransformant by a conventional method, and changes in the expression level of target enzyme protein can be examined by immunoassay (RIA method, ELISA method, FIA method and the like) by using an antibody to the target enzyme. As mentioned above, since most of the gene recombination events in plants result from nonhomologous recombination, the expression of target enzyme does not always decrease in all transgenic plants as compared to wild strains, due to the position effect of chromosome region inserted with the introduced nucleic acid. Therefore, as mentioned above, by measuring and comparing the expression amounts of transformant and untransformant at RNA level or protein level of the target enzyme, lower expression of the target enzyme in a transgenic plant than in a wild strain can be confirmed, based on which the object genetically modified plant can be obtained.

[0041] Plants differentiated from a seed obtained by self-fertilization of the thus-obtained transgenic plants, which show lower expression of the target enzyme as compared to wild strains and, as a result, show higher capsinoid production capability as compared to wild strains are statistically contained at a certain ratio according to the insertion manner of the object nucleic acid. The appearance ratio of the improved capsinoid production capability in plants differentiated from the R1 seed obtained by self-fertilization of a redifferentiated generation (R0) plant generally follows the Mendel's Law. For example, when the object nucleic acid is heterozygously inserted into one gene locus, R1 seeds are divided into 3:1 based on the improved capsinoid production capability. Among the R1 plants differentiated from the R1 seed, those having improved capsinoid production capability are grown and self-fertilized to give R2 seeds. When the improved capsinoid production capability is maintained in all seeds, the R1 plant is determined a homozygote for the introduced nucleic acid, and when the improved capsinoid production capability is shown in 3:1, the R1 plant is determined to be a heterozygote for the introduced nucleic acid. The thus-selected, genetically modified plant which is a homozygote for the introduced nucleic acid is extremely useful as a strain fixedly having an improved capsinoid production capability. Since capsinoid is accumulated in a fruit (fruit skin, placenta and the like), the capsinoid production capability can be known at an earlier stage by desirably using, as an index, expression or activity of a target enzyme which is also expressed in a tissue such as leaf and the like.

[0042] The genetic modification that inhibits activity of an enzyme that catalyzes an amino group conversion reaction from vanillin to vanillylamine is not particularly limited as long as it is known. For example, introduction of an antibody gene to the enzyme, introduction of a dominant-negative mutant gene, and the like can be mentioned.

[0043] To avoid difficulties such as assembly efficiency of H chain and L chain within the plant and the like, the antibody gene desirably encodes a single chain antibody prepared by genetic engineering, such as scFv, scFv-Fc, minibody, diabody and the like. Such antibody can be prepared by producing an antibody producing hybridoma from a mouse immunized with a target enzyme protein or a fragment thereof by a conventional method, cloning the antibody gene from the cell by a conventional method, and ligating the fragment via a linker DNA as appropriate.

[0044] A dominant-negative mutant refers to one with decreased activity due to the introduction of a mutation into a target enzyme. In the dominant-negative mutant, the function of the enzyme can be indirectly inhibited by competitively interacting a target endogeneous enzyme in a wild strain with the substrate vanillin. The dominant-negative mutant can be produced by introducing a mutation into the nucleic acid encoding the target gene. Examples of the mutation include amino acid mutation that causes a decrease in the function provided by a functional site (e.g., deletion, substitution or addition of one or more amino acids). A dominant-negative mutant can be produced by PCR or a method known per se using a known kit.

[0045] As a method of introducing a DNA encoding an antibody gene and dominant-negative mutant into a target plant to give a transgenic plant, a method similar to the above-mentioned methods for introducing nucleic acid containing a nucleotide sequence complementary or substantially complementary to the nucleotide sequence of mRNA of a target enzyme, or a part thereof, is preferably used.

[0046] Therefore, by measuring and comparing the activity of the target enzyme between transformant and untransformant, lower activity of the target enzyme in the obtained genetically modified plant than in a wild strain can be confirmed, based on which the object genetically modified plant can be obtained. For example, when the target enzyme is pAMT, a protein is extracted from placenta, flower and the like, vanillin is added to the extract and the mixture is incubated for a given time, and the resulting vanillylamine is quantified by HPLC and the like, whereby the enzyme activity can be examined.

[0047] The genetically modified plant obtained as mentioned above, which shows a decreased expression or activity of an enzyme that catalyzes an amino group conversion reaction from vanillin to vanillylamine as compared to a wild strain, shows increased capsinoid production capability as compared to the wild strain. The "increased production capability" may be further increased capsinoid production capability as compared to a wild strain having an ability to produce capsinoids by nature, or production capability newly acquired by a plant incapable of producing capsinoid by nature.

[0048] The present invention also provides a production method of capsinoids, including recovering capsinoids from the genetically modified plant of the present invention. As a method of recovering capsinoids, any known method may be used and, for example, the methods described in the above-mentioned patent document 1 (JP-A-11-246478), JP-A-2002-226445, JP-A-2004-018428, W02005/122787 and/or 2006/043601 can be used unlimitatively. Examples

Example 1: Production of pAMT gene expression-suppressive transformed chili pepper

[0049] pAMT gene amplified by RT-PCR with cDNA extracted from CH-19 Sweet was inserted into a GUS region of Ti-plasmid vector pIG121-Hm in the antisense direction (Fig. 2). The plasmid was introduced into Agrobacterium tumefaciens EHA101 strain and used for transformation of chili pepper.
The surface of a seed of Capsicum annuum variety 'Takanotsume' was sterilized with 70% ethanol for 1 min and 2% sodium hypochlorite for 15 min, rinsed 3 times with sterile water, and sown in MS medium. Cotyledon germinated 10 days later was cut and transplanted to MS medium containing 10 mg/l benzyladenine (BA). 16 hr day-length culture was performed at 25°C for 24 hr, and the cotyledon was immersed for 10 min in Agrobacterium suspension cultured in YEP medium containing 50 mg/l kanamycin and 50 mg/l hygromycin for 24 hr. To remove redundant Agrobacterium suspension, the leaf was placed on sterilized filter paper, and then transplanted again to MS medium containing 10 mg/l benzyladenine (BA). After co-cultivation in a dark place for 3 days, the leaf was transplanted to MS medium (selection medium) containing 10 mg/l BA, 50 mg/l kanamycin and 300 mg/l carbenicillin, and 16 hr day-length culture was performed at 25°C. To suppress growth of Agrobacterium, the selection medium was exchanged with a new medium every 10 days - 2 weeks. A shoot redifferentiated 1 - 2 months from the start of the selection was transplanted to MS medium (rooting medium) containing 50 mg/l kanamycin and 300 mg/l carbenicillin to induce rooting. The rooted plants of 16 strains were transplanted to soil, and grown in phytotron. DNA was extracted from leaf/leaves of the selected individual, and genomic PCR analysis was performed using a primer that amplifies NPTII gene in the vector. The PCR product was applied to 1.5% agarose gel electrophoresis, and stained with ethidium bromide. As a result, the selected individual was found to have NPTII gene introduced thereinto (Fig. 3).

Example 2: Measurement of capsiate and capsaicin contents of transformed chili pepper

[0050] Fruits were taken from 16 strains of transformed chili pepper and Capsicum annuum variety 'Takanotsume' without introduction of a gene, and capsiate and capsaicin contents were measured by a conventional method. As a result, 3 transformed chili pepper fruits (TG-3, 8 and 14) were found to contain capsiate in larger amounts than in control Capsicum annuum variety 'Takanotsume'. TG-3 and TG-8 had lower capsaicin contents than other strains (Fig. 4(a) and 4(b)).

Example 3: Transcription and expression analysis of pAMT gene in transformed chili pepper fruit

[0051] RNA was extracted from a fruit of a strain with higher capsiate content, and RT-PCR analysis of transcription amount of endogenous pAMT gene was performed. As a result, the strain with high capsiate content was found to show suppressed transcription and expression of pAMT gene. On the other hand, in a strain wherein the capsiate content was not high even though the gene had been introduced, the transcription and expression of endogenous pAMT gene was not suppressed (Fig. 5). From the above results, it has been found that a plant can be modified to artificially synthesize capsiate, by suppression of expression of pAMT gene.

Example 4: Analysis of pAMT protein amount in transformed chili pepper fruit

[0052] The full-length pAMT gene isolated from CH-19 Hot was inserted into a multicloning site of Escherichia coli expression vector (pColdI vector, Takara Bio Inc.). The obtained vector was transformed with Chaperon Competent Cell BL21 (Takara Bio Inc.) and cultured with shaking in LB medium (500 ml) containing 20 mg/l chloramphenicol, 50 mg/l ampicillin, 10 μl/l tetracycline and 1 g/l L-arabinose for 10 hr at 37°C. When the culture medium showed OD600 of 0.6, 1 mM IPTG was added and the mixture was cultured with shaking at 15°C for 24 hr.
The grown E. coli was recovered by centrifugation, and subjected to protein extraction and Histag purification using QIAexpress Ni-NTA Fast Start Kit (QIAGEN). The purified protein was confirmed by SDS-PAGE. The protein was injected to a rabbit to produce a polyclonal antibody.
Using the produced polyclonal antibody, the protein extracted from the fruit was electrophoresed by 1 mg SDS-PAGE and analyzed by Western blot. As a result, TG-3 and TG-8 that showed accumulation of capsiate showed a remarkably decreased pAMT protein amount. Also in TG-14, the pAMT protein amount was lower than CH-19 HOT (Fig. 6).
[0053] From the foregoing, it has been shown that suppression of expression of pAMT gene of chili pepper that biosynthesizes capsaicinoids enables production of a plant showing an increased amount of capsinoid biosynthesis.

Industrial Applicability

[0054] According to the present invention, capsinoids can be highly produced from a plant conventionally incapable of producing capsinoids or capable of producing capsinoids in a low amount. The present invention is useful since it can provide capsinoids, which are expected to be applicable to various fields such as medicines, health foods and the like, in large amounts at a low cost.

[0055] This application is based on patent application No. 2008-163884 filed in Japan (filing date: June 23, 2008), the contents of which are encompassed in full herein.

SEQUENCE LISTING

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<120>  Capsinoid Biosynthesizing Genetically Modified Plants

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    370                 375                 380

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```

Claims

1. A genetically modified plant capable of producing capsinoids, which shows a decreased expression or activity of an enzyme that catalyzes an amino group conversion reaction from vanillin to vanillylamine as compared to wild strains.

2. The plant according to claim 1, wherein the aforementioned enzyme is a pAMT gene product.

3. The plant according to claim 1 or 2, wherein the aforementioned decreased expression or activity of the enzyme is caused by a destruction or mutation of the enzyme gene, a decomposition or suppressed translation of a transcription product of the gene, or inhibition of action of the enzyme on vanillin.

4. The plant according to any one of claims 1 to 3, wherein the wild strain has a capsaicinoid biosynthesis system.

5. The plant according to claim 4, wherein the wild strain is a plant belonging to the genus Capsicum.

6. A method of producing a genetically modified plant capable of producing capsinoids, comprising performing a genetic modification to decrease expression or activity of an enzyme that catalyzes an amino group conversion reaction from vanillin to vanillylamine in a plant having a capsaicinoid biosynthesis system.

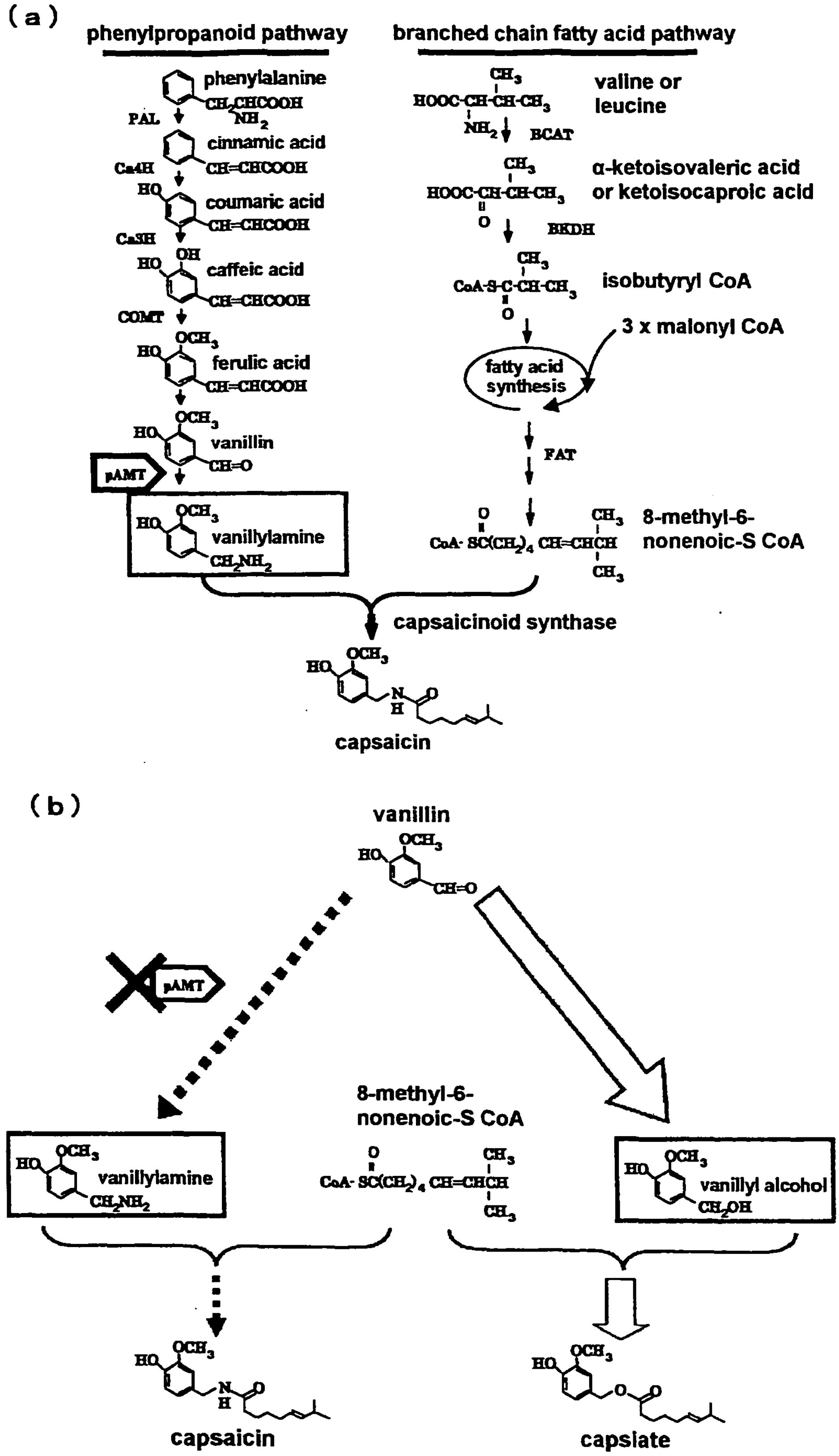
7. The method according to claim 6, wherein the genetic modification is selected from the group consisting of an introduction of DNA encoding an antisense RNA, iRNA, ribozyme or dominant-negative mutant for the aforementioned enzyme gene, a destruction of the gene by a knockout method or mutagen treatment, and an introduction of a gene encoding an antibody against the enzyme.

8. The method according to claim 6 or 7, wherein the aforementioned enzyme is a pAMT gene product.

9. The method according to any one of claims 6 to 8, wherein the plant having a capsaicinoid biosynthesis system belongs to the genus Capsicum.

10. A method of producing capsinoid, comprising recovering the capsinoid from a fruit of the plant according to any one of claims 1 to 5.

FIG. 1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ferulic acid
vanillin
pAMT
vanillylamine
valine or
leucine
BCAT
α-ketoisovaleric acid
or ketoisocaproic acid
BKDH
isobutyryl CoA
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fatty acid
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FAT
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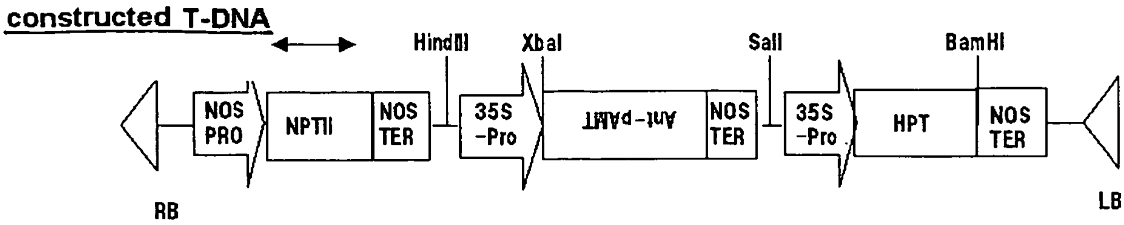
FIG. 2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RB
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FIG. 3

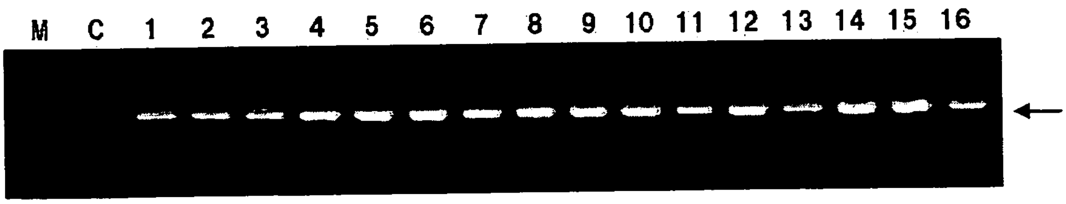
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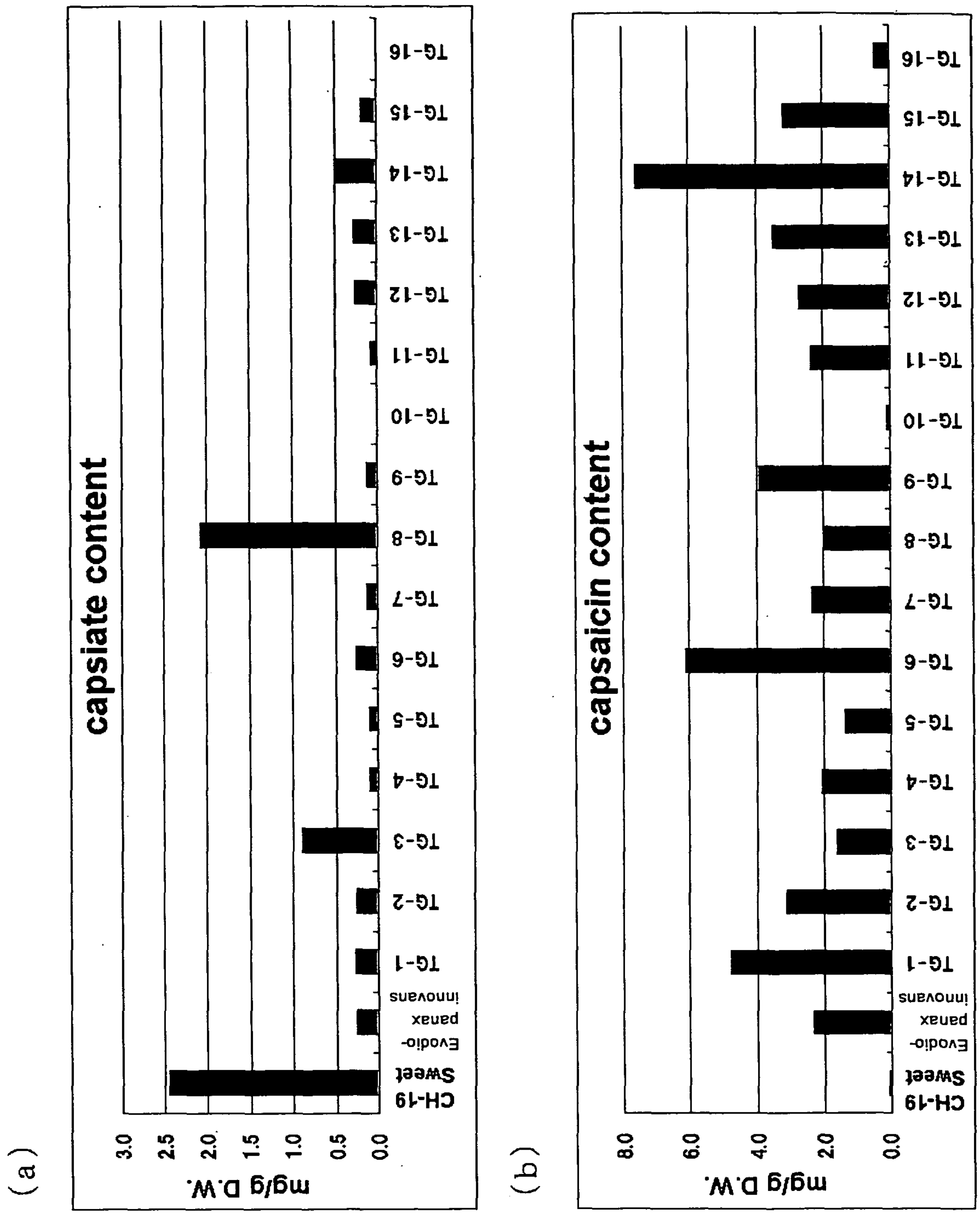
FIG. 4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2.0
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1.0
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Evodio-panax innovans
TG-1
TG-2
TG-3
TG-4
TG-5
TG-6
TG-7
TG-8
TG-9
TG-10
TG-11
TG-12
TG-13
TG-14
TG-15
TG-16
(b)
capsaicin content
mg/g D.W.
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6.0
4.0
2.0
0.0
CH-19 Sweet
Evodio-panax innovans
TG-1
TG-2
TG-3
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FIG. 5

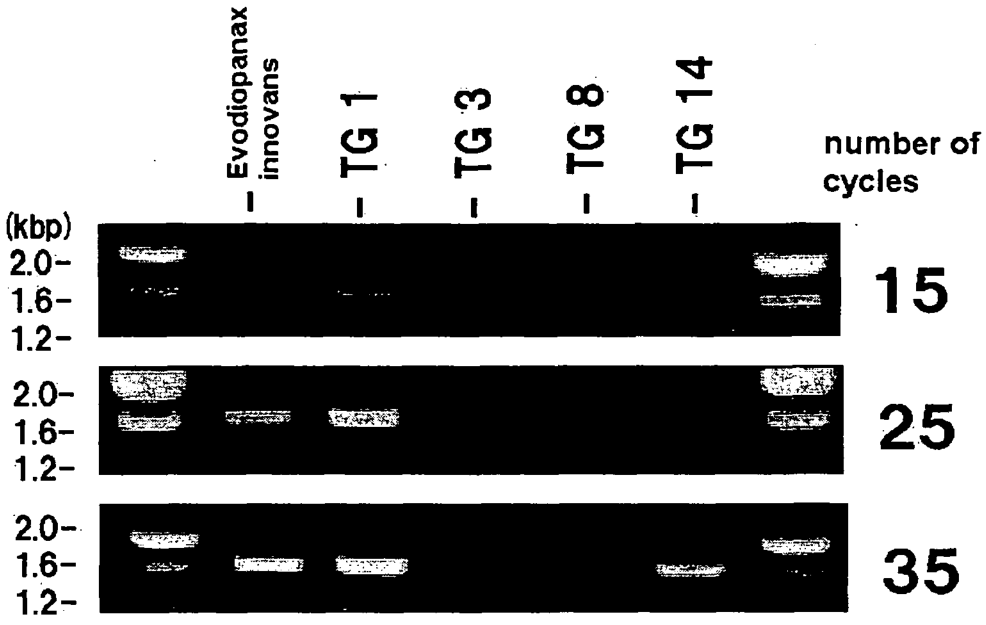
Evodiopanax innovans
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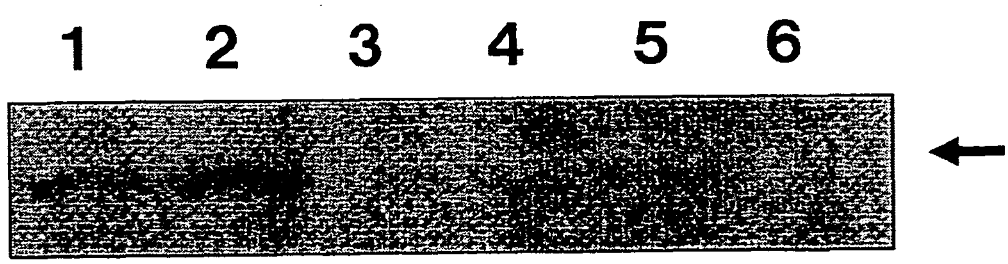
FIG. 6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INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2009/061169

A. CLASSIFICATION OF SUBJECT MATTER
A01H5/00(2006.01)i, *C12N15/09*(2006.01)i, *C12P7/62*(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A01H5/00, C12N15/09, C12P7/62

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Jitsuyo Shinan Koho	1922-1996	Jitsuyo Shinan Toroku Koho	1996-2009
Kokai Jitsuyo Shinan Koho	1971-2009	Toroku Jitsuyo Shinan Koho	1994-2009

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
JSTPlus/JMEDPlus/JST7580(JDreamII), CA/BIOSIS/MEDLINE(STN), WPI, PubMed, CiNii,G-Search, Shokuhin Kanren Bunken Joho (Shoku Net),

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X/A	del Rosario Abraham-Juarez M., et al., Virus-induced silencing of Comt, pAmt and Kas genes results in a reduction of capsaicinoid accumulation in chili pepper fruits., Planta, 227(3)(2008.2), p.681-695	1-9/10
X/A	Kobata K., et al., Novel Capsaicinoid-like Substances, Capsiate and Dihydrocapsiate, from the Fruits of a Nonpungent Cultivar, CH-19 Sweet, of Pepper (Capsicum annuum L.), J. Agric. Food Chem., 46(5)(1998), p.1695-1697	1-5,10/6-9

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 July, 2009 (21.07.09)	04 August, 2009 (04.08.09)

Name and mailing address of the ISA/	Authorized officer
Japanese Patent Office	
Facsimile No.	Telephone No.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2009/061169

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	Lang Y., et al., Functional loss of pAMT results in biosynthesis of capsinoids, capsaicinoid analogs, in Capsicum annuum cv. CH-19 Sweet., Plant J. [online], Epub 18 May 2009, [retrieved on 17 Jul 2009], Retrieved from the Internet: <URL:http://www3.interscience.wiley.com/journal/122389101/abstract>	1-10

Form PCT/ISA/210 (continuation of second sheet) (April 2007)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2009/061169

Continuation of B. FIELDS SEARCHED
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Foods Adlibra/Foodline/FSTA(DIALOG),

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REFERENCES CITED IN THE DESCRIPTION

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- JP 11246478 A **[0006] [0048]**
- JP 2001026538 A **[0006]**
- JP 2002226445 A **[0048]**
- JP 2004018428 A **[0048]**
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- **Yazawa et al.** *Journal of the Japanese Society for Horticultural Science,* 1989, vol. 58, 601-607 **[0007]**
- *Biosci. Biotech. Biochem.,* 2001, vol. 65 (12), 2735-2740 **[0007]**
- Current Protocols in Molecular Biology. John Wiley & Sons, 1999, 6.3.1-6.3.6 **[0024]**
- *Nature,* 2001, vol. 411 (6836), 494-498 **[0029]**
- *Proc. Natl. Acad. Sci. USA,* 2001, vol. 98 (10), 5572-5577 **[0030]**
- *Nucleic Acids Res.,* 2001, vol. 29 (13), 2780-2788 **[0030]**